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Gene Targeting the *myf*-5 Locus With *nlacZ* Reveals Expression of This Myogenic Factor in Mature Skeletal Muscle Fibres as Well as Early Embryonic Muscle

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ABSTRACT **We have introduced the *nlacZ* reporter gene into the locus of the myogenic factor gene *myf-5* by homologous recombination in embryonic stem (ES) cells. Targeted ES clones were injected into precompaction morula, and the β-galactosidase expression pattern was monitored. These mice permit the sensitive visualization of *myf-5* expression throughout the embryo, and provide a standard for comparing it with that seen with different *myf-5/nlacZ* transgenes. Thus, in a comparison using ES cells in chimaeric embryos containing the targeted or randomly integrated *myf-5/nlacZ* construct, we demonstrate that 5.5 kbp of *myf-5* upstream flanking sequence including exon1 and most of intron1 directs some skeletal muscle expression, but this is neither qualitatively nor quantitatively equivalent to that of the endogenous gene. *Myf-5* is expressed early, before terminal myogenesis takes place in the medial half of the somite, and subsequently it is a major myogenic factor as skeletal muscle forms. All skeletal muscle shows β-galactosidase activity, even after birth, indicating that *myf-5* expression is not confined to primary myotubes, which are derived from embryonic myoblasts, but is also present in muscles containing different adult fibre types. The presence of myf-5 transcripts from the endogenous gene in older muscle was confirmed by in situ hybridization. These results suggest that the *myf-5* gene is not activated in only a subset of muscle cells and are consistent with the results on the MyoD knockout mice.** © 1996 Wiley-Liss, Inc.

Key words: *myf-5*, Homologous recombination, Mature skeletal muscle, Transgene

INTRODUCTION

Muscle is a mesodermal derivative which becomes established from the time of gastrulation (Bellairs et al., 1986). In the head, craniofacial muscles are derived from the cephalic prechordal and paraxial mesoderm (Couly et al., 1992; McClearn and Noden, 1988; Noden, 1983a,b; Wachtler et al., 1984) as well as from the most anterior somites (Noden, 1983a). All skeletal muscles in the trunk are derived from the somites, which result from segmentation of the anterior portion of the segmental plate (paraxial) mesoderm and form in pairs on either side of the neural tube following a rostrocaudal developmental gradient. The dorsal compartment differentiates to give the dermomyotome (dermis and muscle), and the ventral compartment gives the sclerotome (axial skeleton). The first skeletal muscle of the body forms as muscle precursor cells located cranially in the dorsomedial part of the dermomyotome, immediately adjacent to the neural tube, migrate underneath the dermomyotome layer to form the myotome in the central region of the somite (Ede and El-Gadi, 1986; Kaehn et al., 1988). In the mouse this takes place from embryonic day 8.5 (E8.5). These myotomal cells do not subsequently migrate out from the somite, but will later contribute to axial musculature (epaxial) on either side of the vertebral column. All other skeletal muscles of the body located peripherally, such as the intercostals or body wall (hypaxial) or those in the limb, arise by migration of muscle precursor cells from the ventrolateral half of the dermomyotome (Chevallier et al., 1977; Christ et al., 1977; Ordahl and Le Douarin, 1992). The same mechanisms for the formation of myotomal and peripheral body musculature probably operate in birds and mammals, but in the latter, the location of precursor cells in the somite is less clearly defined (Milaire, 1976).

Shortly after myotome formation, multinucleate primary fibres begin to appear both in the trunk and the limbs. By E14.5 in the mouse, secondary fibres begin to accumulate adjacent to the primary muscle scaffolds, and can be distinguished by their morphology (Ontell and Kozeka, 1984) and the contractile protein isoforms present. Primary and secondary fibres are thought to arise from embryonic and foetal myoblasts, respectively, and these muscle precursors can be distinguished in culture (Cossu et al., 1993). It is not clear whether foetal myoblasts are derived from a different population of precursors or whether they correspond to a proportion of embryonic cells which remained quies-

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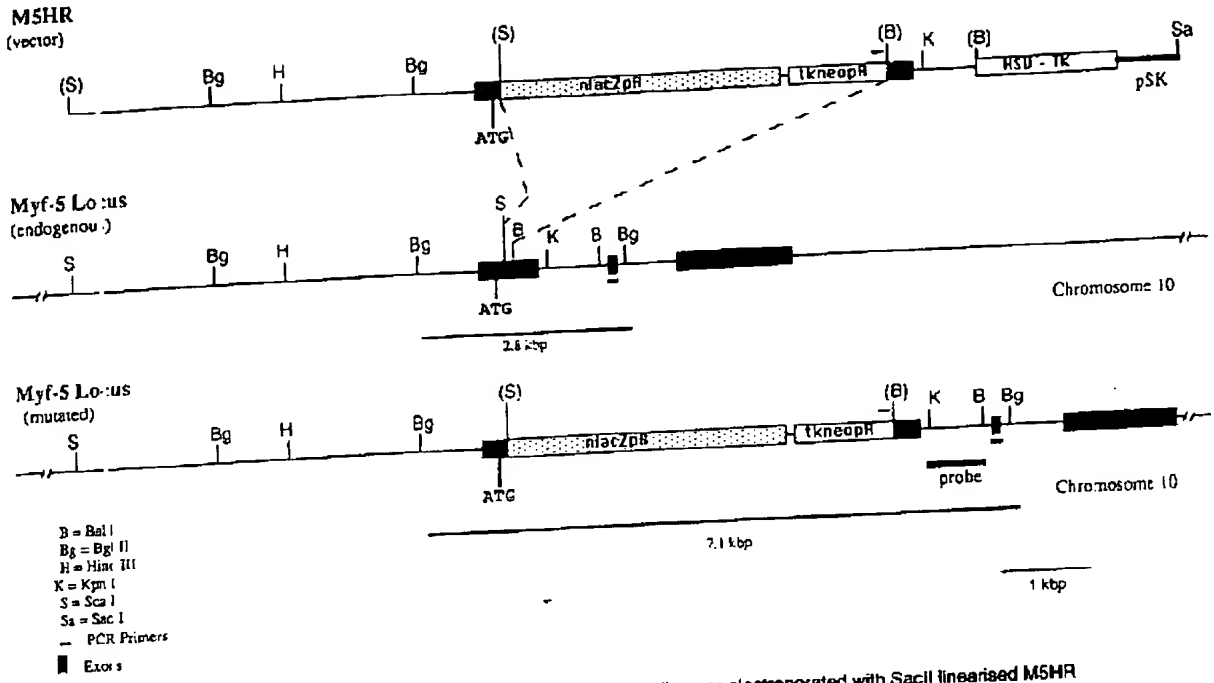


Fig. 1. Strategy for targeting the *myf-5* locus. D3 ES cells were electroporated with SacII linearised M5HR vector. Primers located in the *Neo* gene and outside the region of homology were used for PCR identification of clones. The 3' *myf-5* fragment was used as a probe against BglII digested ES cell DNA.

TABLE 1. Summary of Electroporations With the *myf-5* Targeting Construct

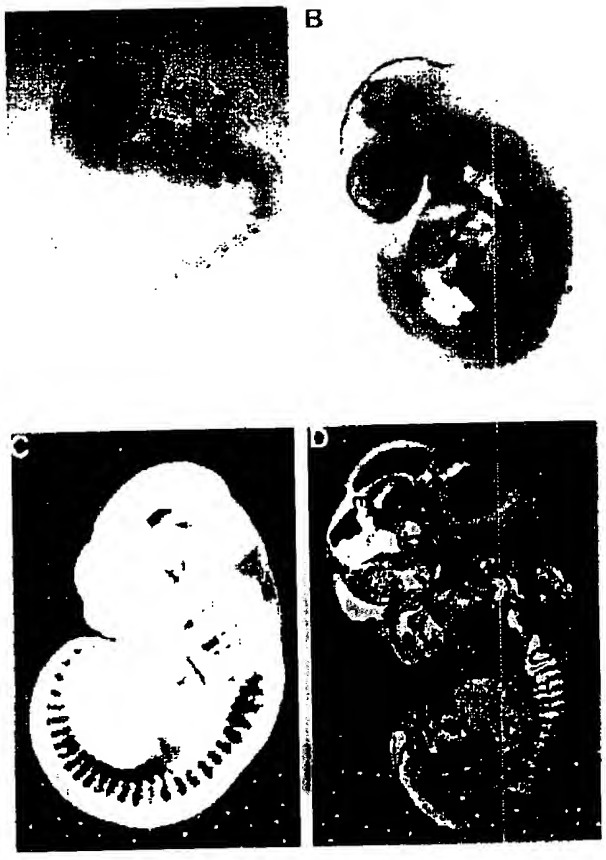
	Cells electroporated	$G418^R/GANC^R$	TK enrichment	PCR clones	Freq. G418 + GANC[a]	Theoretical freq. G418[b]
I	2×10^7	230 (180)	14	1	1/180	1/2,520
II	2×10^7	277	11	2	1/139	1/1,524
IV	1.8×10^7	434	9	0	0/434	0/3,609
V	2×10^7	444	5	2	1/222	1/1,110
VI	2×10^7	360	5	2	1/180	1/900
VII	2×10^7	351	6	1	1/351	1/2,100
Total		2,046	8.3 avg.	8		

[a]Average frequency $G418^R$ + $GANC^R$: 1/255.
[b]Average theoretical frequency $G418^R$: 1/2116.

cent initially (Cusella-De Angelis et al., 1994). Foetal myoblasts may acquire distinct properties as a result of the massive cell division which they undergo, just before differentiating into secondary fibres (Harris et al., 1989). Before birth the adult fibre types characteristic of different fast and slow muscles begin to appear; slow fibres are mainly derived from the primary fibre population, whereas most secondary fibres mature to give different fast fibre types, following the electrical activity of the motor neuron (see Kelly, 1987).

Experiments with skeletal muscle cells in culture have shown that the activation of many muscle genes depends on the MyoD family of bHLH transcription factors which in mammals consists of MyoD, myogenin, MRF4, and *myf-5* (Weintraub et al., 1991). In situ hybridization studies have shown that each of these factors has a distinct pattern of expression during skeletal myogenesis, and *myf-5* is the only member to be transcribed prior to muscle formation in the mouse (Buckingham, 1992). These transcripts are present in the immature somite and accumulate in the dorsomedial part of the dermomyotome prior to myotome formation (Buckingham, 1992; Ott et al., 1991). They are also detected relatively early in the limb buds and in other peripheral pre-muscle masses. In myotomal muscle from about E10.5, the *MyoD* gene is activated. In foetal muscle, *myf-5* transcripts were not detected after E14; MyoD and myogenin are the major myogenic factors present, to be replaced by MRF4 as the predominant factor in adult skeletal muscle. Little is known about

[illegible] Southern analysis of targeted *myf-5* ES clones. Tail DNA from PCR-positive ES clones was digested with BglII and hybridized with the probe indicated in Figure 1. The endogenous (7.1 kb) and targeted (2.8 kb) *myf-5* bands are indicated. **Lane 1** contains normal DBA/2 liver DNA digested with BglII.



[illegible] Expression pattern of *myf-5* in chimeric embryos. (A) Whole-mount [illegible] embryo. The arrowhead points to the presumptive [illegible] at the level of the forelimb bud. (B) Lateral view of the head region of the embryo shown in A. (C) Transverse section through the trunk region of another CNS-positive embryo. The arrowhead points to the myotome. Note the absence of the medial region of the myotome. (D) Transverse section through the head region of another CNS-positive embryo. The arrowhead points to the [illegible] muscle. Note the absence of the tongue muscle. Other [illegible] are also apparent.

the regulation of the *myf-5* gene, which lies 5.5 kbp downstream of the *MRF4* gene (Miner and Wold, 1990). A *lacZ* transgene under the control of this intergenic region only partially reproduces the endogenous expression pattern (Patapoutian et al., 1995; present data). Gene knock-out experiments have shown that either *myf-5* (Braun et al., 1992) or MyoD (Rudnicki et al., 1992) alone, skeletal muscle will form. In the absence of *myf-5* myotome is lacking initially, but *MyoD* is activated 2 days later and subsequently different trunk and limb muscles appear. *Myf-5*$^{-/-}$ mice die perinatally with a ribs deficiency, which may be an indirect result of the lack of early myotome (Braun et al., 1992). *MyoD*$^{-/-}$ mice express high levels of *myf-5*, have normal skeletal muscle fibres, and are viable. An alternative explanation that counters the presumptive myoblast populations and then expression of myogenic factors (Braun and Arnold, 1995) myoblasts normally express only *MyoD*, and the *myf-5* population is lacking in the *MyoD*$^{-/-}$ mouse is complemented by the expansion of a *myf-5*$^{+}$ embryonic myoblast population. Alternatively do both embryonic and fetal myoblast populations express *myf-5*?

In order to address these questions and to exploit *myf-5* expression as a useful lineage marker for myoblasts and their precursors, we have introduced the *Escherichia coli* β-galactosidase (*lacZ*) coding sequence into the *myf-5* gene by homologous recombination so that expression of β-galactosidase is under the control of the endogenous *myf-5* locus. This approach assures that all regulatory sequences are present, which is often not the case with transgenic reporters for β-galactosidase are very sensitive and give resolution at the single cell level. Our analysis of *myf-5*/*lacZ* expression and fate mapping experiments has permitted us to look in greater detail at the expression of this gene during myogenesis. We report on the location of *myf-5*$^{+}$ (*lacZ*$^{+}$) cells in the somite before and during early muscle formation, and also on the presence of these cells in all types of mature skeletal muscle. This finding clearly demonstrates that *myf-5* expression is not restricted to a subpopulation of embryonic myoblasts.

RESULTS

The gene targeting strategy used is shown in Figure 1. A positive-negative selection vector, MSHR, containing 5.6 kbp (5′) and 1.05 kbp (3′) of the *myf-5* locus flanking the *nlacZ* and *PGKneo* genes. The *nlacZ* gene is fused

into the first exon of the *myf-5* gene, 13 amino acids after the ATG codon such that the *nlacZ* coding sequence is in phase for translation. Therefore, translation of the β-galactosidase protein probably reflects translational as well as transcriptional regulation of the endogenous *myf-5* gene. The *myf-5* gene itself is disrupted, and a small deletion is introduced.

Six electroporations were carried out, and the results of each experiment are summarized in Table 1. After G418 and gancyclovir (GANC) selection, over 2,000 surviving clones were analysed. The average targeting frequency was 1/2,116 in G418 clones and 1/256 for G418-GANC doubly resistant clones. The average enrichment using GANC was 8.3-fold. A total of eight clones were isolated using a PCR screen, and six of these were analysed by Southern blotting (Fig. 2). A BglII digest of these clones reveals the endogenous 2.8-kbp allele and a 7.2-kbp targeted allele. More extensive analysis and hybridization with the *Neo* gene showed that these clones were correctly targeted at the 5′ and 3′ ends and contained only one copy of the targeting vector (data not shown).

Several of the targeted ES lines were used for injections into precompaction morula to generate chimaeric embryos. All the lines tested showed a similar pattern of expression. Highly chimaeric embryos were obtained by this approach (Conquet, 1991; Lallemand and Brûlet, 1990; and our unpublished observations). Muscle chimaerism was estimated as up to 90%, by examination of β-galactosidase–stained nuclei in the muscle masses.

Sites of *myf-5* Expression in the Developing Embryo

Figure 3 shows whole mount coloration of embryos obtained at E8.5, E9.5, and E10.5. This corresponds to the period when *myf-5* transcripts are detected in the somites and peripheral muscle masses by in situ hybridization. β-galactosidase–positive (β-gal$^+$) cells are clearly present in the somites at these stages. At E8.5, before turning of the embryo, the first somites are labelled. In older embryos, some β-gal$^+$ cells are also detectable in the immature caudal somites. We have been able to detect β-gal$^+$ cells in the penultimate caudal somite adjacent to the neural tube. The first rostral somite to be labelled is a half-somite (Ede and El-Gadi, 1986) which labels to about half the intensity of the subsequent somite (Fig. 3A). As development proceeds expression of β-galactosidase is seen in myotomes and in the sites where peripheral muscle masses will form, following the rostrocaudal developmental gradient. At E9.5 (Fig. 3B) muscle precursor cells which express *myf-5* are already present in the developing temporalis and extraocular muscles. The latter muscles are formed by a number of component muscles which originate from both the cephalic prechordal and paraxial mesoderm (Adelman, 1927; Couly et al., 1992; Noden, 1983a; Wachtler et al., 1984; Wachtler and Jacob, 1986). At this stage, the branchial arches which will give rise to the facial muscles are well developed. The (first) mandibular arch is labelled by E9 followed by the (second) hyoid arch and the third visceral arch. By E10.5 (Fig. 3C) the hyoid arch is the most intensely labelled. These muscle cells are confined to the inner core of the arches and originate from cephalic prechordal mesoderm (Seifert and Christ, 1990). The surrounding cells comprising the remainder of the arch are neural crest derived (Noden, 1988).

At E10.5 the forelimb bud contains β-gal$^+$ cells, although the hindlimb bud is still negative. At E10.5 for the hindlimb bud (Fig. 3C) and at E9.5 for the forelimb (Fig. 3B), we see no evidence of β-gal$^+$ cells in the field between limb bud and adjacent somites, as expected from our experiments with limb bud explants from such mice which clearly show that *myf-5* is only activated once the cells have migrated (Tajbakhsh and Buckingham, 1994). We have recently confirmed these findings with heterozygote embryos (S.T., unpublished observations). Labelling at this stage in the myotome is due to a dorsal epaxial component and to newly forming intercostal and body wall muscle masses (hypaxial muscles) which appear immediately ventral to and distinct from the epaxial muscles, and which have originated from the ventrolateral edge of the dermomyotome. This is clearly seen also in the posterior portion of the forelimb, and can mistakenly be interpreted on sections as cells "migrating" to the limb (Fig. 3C). A striking band of β-gal$^+$ cells originating from the occipital somites (both sides of the embryo) and crossing the anterior surface of the heart is present. By E11 this β-galactosidase labelling becomes restricted to either side of the throat spanning the first two arches (data not shown). This band corresponds to muscle cells of the hypoglossal cord and those that will subsequently contribute to muscles of the tongue (Hunter, 1935; Noden, 1983a). By E12.5 there is extensive labelling of all skeletal muscles. Head muscles, whether derived from the first somites or from cephalic prechordal or paraxial mesoderm, are labelled, as are muscles throughout the body. This point is illustrated in Figure 3D showing a sagittal section of a E12.5 embryo taken with dark-field optics where the β-galactosidase staining appears pink. It demonstrates labelling in temporalis, jaw, intercostal, and rostrally located back muscles, the latter being epaxial. A β-gal$^+$ caudal myotome is also visible. The first muscle cells which will contribute to the diaphragm are detectable at this stage immediately anterior to the liver.

No β-gal$^+$ cells are detected in the heart, either in the myocardium or in the conduction systems. The fact that the heart tube is negative before E8 (data not shown), makes it unlikely that the *myf-5* gene is transiently expressed during cardiogenesis. Similarly, no label is detected in smooth muscle as it forms in the embryo. β-galactosidase–labelled cells are, however, seen at a few discrete sites in addition to bona fide skeletal muscle and its precursors. Although the oesophagus originates as a smooth muscle derivative,

fous tissue will eventually be composed of skeletal muscle in the late foetal stage and continuing after birth (see Patapoutian et al., 1995). Indeed, β-gal$^+$ cells are clearly seen in the oesophagus after birth. From E16.5, some *myf-5*$^+$ cells are also seen in the thymus, becoming prominent after birth (Fig. 4D). Since the myoid cells of the thymus have myogenic potential (see Discussion), this finding is not unexpected. Totally unexpected, however, is the finding of *myf-5*$^+$ cells in the central nervous system (CNS) (Fig. 3). The localisation of β-gal$^+$ cells in regions of the mesencephalon and rhombencephalon is demonstrated in the sagittal section (Fig. 3D; see Tajbakhsh and Buckingham, 1995).

Embryonic Expression of a *myf-5* Transgene

Embryos with the *myf-5* gene targeted by *nlacZ* provide a standard against which the expression of transgenes containing potential *myf-5* regulatory sequences can be measured. Three independent ES cell lines which had randomly integrated the construct shown in Figure 1 were also microinjected into morulae to follow their expression pattern. One line showed ectopic expression throughout the neural tube which was distinct from that seen previously for *myf-5*; ectopic expression is probably a result of regulatory sequences present at the site of integration. In all three lines, labelling of skeletal muscle was seen, but it was quantitatively much less than that seen with the endogenous gene (Fig. 5). β-gal$^+$ cells were detected in the somites, initially in the arches, but not in limb buds or trunk. Some ectopic expression was seen in the head mesenchyme lateral to the eye. We conclude from these experiments that the 5.5-kbp flanking fragment containing the *myf-5* promoter and the *MRF4*-*myf-5* intergenic region, in addition to exon 1 and most of intron 1, are not sufficient to reproduce the pattern of *myf-5* expression in the embryo.

Myf-5 in Foetal and Post-Natal Skeletal Muscles

Myf-5 is a major myogenic factor in embryonic skeletal muscle to be replaced later by Myo D (Buckingham, 1992). However, at E14.5 all skeletal muscles continue to be labelled with β-galactosidase (Fig. 4A–C), a stage at which secondary muscle fibre formation has been initiated in many of the more ventral muscle masses. In the hindlimb, neuromuscular junctions are forming, and changes in gene expression such as the accumulation of myosin MLC3F transcripts, have been documented in detail (Ontell et al., 1988). In the forelimb, by E14.5, these events are more advanced, and secondary myotubes are already forming. Sections through the forelimb, trunk, and head regions of an E14.5 mouse embryo were examined by in situ hybridization for expression of the endogenous *myf-5* gene. Although the signal is lower than at E11.5 with a double-labelled probe and longer exposure times, *myf-5* transcripts are still clearly detectable throughout the muscle masses, as seen in the hindlimb at late foetal stages (Fig. 6D).

Analysis of newborn mice indicates that β-galactosidase continues to be present in all skeletal muscles with no distinction between fibre types (Fig. 4B,C,E). In the limb (Fig. 4E), most of the skeletal fibre masses are of the fast (IIA, IIB, IIC) type with some slow (type I) fibres (Kelly, 1987; Schiaffino and Reggiani, 1995), whereas in the diaphragm (Fig. 4C) type I and type IIX fibres predominate (DeNardi et al., 1993). We interpret these results to reflect continued low level transcription of the *myf-5* gene in all skeletal muscles at later stages of development. In Figure 7, sections through the hindlimbs of postnatal mice are shown. In this case these were heterozygote animals. It is clear that nuclei in the different muscles are labelled with β-galactosidase. Since skeletal muscle fibers are multinucleate, we asked whether all nuclei are labelled with β-galactosidase. Double labelling with Hoechst 33258 and staining for β-galactosidase suggested this to be the case (data not shown). Labelling decreased within the first week after birth (Fig. 7).

DISCUSSION

Targeted Versus Transgenic Expression of *myf-5*:*nlacZ*

Incorporation of the *nlacZ* reporter into the *myf-5* locus by homologous recombination in ES cells has permitted us to track cells expressing this early myogenic marker. Our observations with mice generated from these cells show the expected expression pattern of *myf-5* at all sites previously documented, in addition to providing new information about further unexpected sites of expression, which have since been confirmed for the endogenous gene by in situ hybridization. Therefore there is no reason to suppose that the insertion in the first exon of the *myf-5* gene has perturbed any endogenous regulatory elements. Mice with an *nlacZ* targeted *myf-5* gene provide a quantitative standard to measure the efficiency with which genomic fragments from the *myf-5* locus direct expression of the same reporter sequence in transgenic experiments. With 5.5 kbp of 5′ flanking sequence and including exon 1 and most of intron 1, the level of expression of the transgene is strikingly reduced. Since injection of ES cells into morulae gives highly chimaeric embryos, and hence chimaeric muscles, as estimated by the percentage of β-gal$^+$ nuclei, this is unlikely to be due to chosen level of chimaerism. This approach for constructing transgenic animals has some advantages which result from the availability of the ES clone. First, multiple transgene embryos with the same integration site can be screened for analysis. Second, a permanent transgenic line can potentially be generated from the ES clone used to make the chimaeras. Third, the ES clone can be differentiated in vitro to provide additional information. As far as the regulation of the *myf-5* gene is concerned, our results are in agreement with a previous report (Patapoutian et al., 1995) with the 5.5-kbp flanking sequence alone, which also showed qualitative differences in the onset of expression of this transgene in

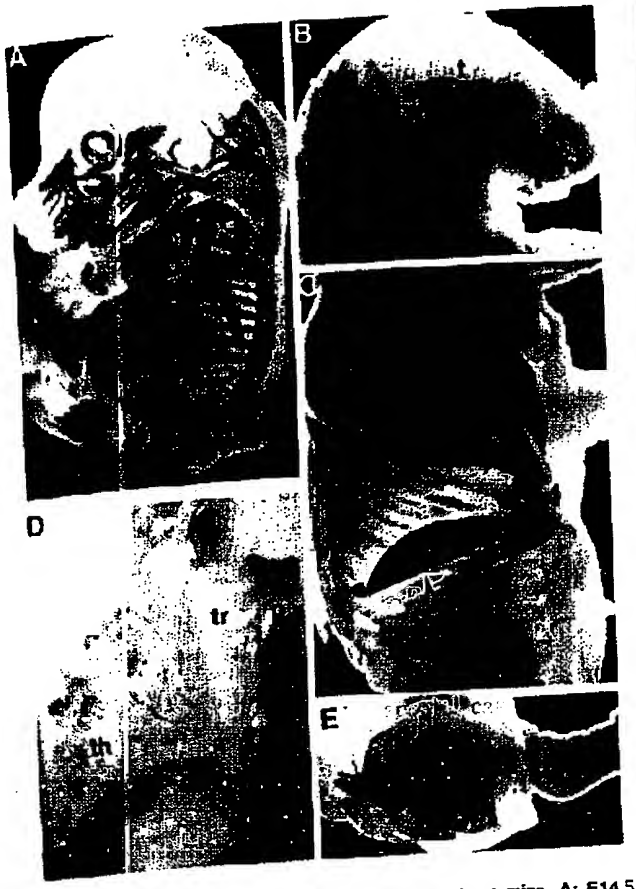


Fig. 4. Expression of *myf-5* in foetal and newborn mice. **A:** E14.5 heterozygote embryo showing extensive labelling of all skeletal muscles. The embryo was fixed and stained for β-galactosidase then cleared with KOH and glycerol. **B:** Head of chimaeric newborn mouse showing expression in the temporalis (t), facial, and neck muscles. The skin was removed prior to staining. **C:** Trunk of newborn mouse; the diaphragm is indicated (arrowhead). **D:** Coloration of oesophagus (o), and thymus (th) of a postnatal day 2 mouse (P2). Trachea (tr). **E:** Hindlimb of newborn mouse showing labelling in all the muscle masses.

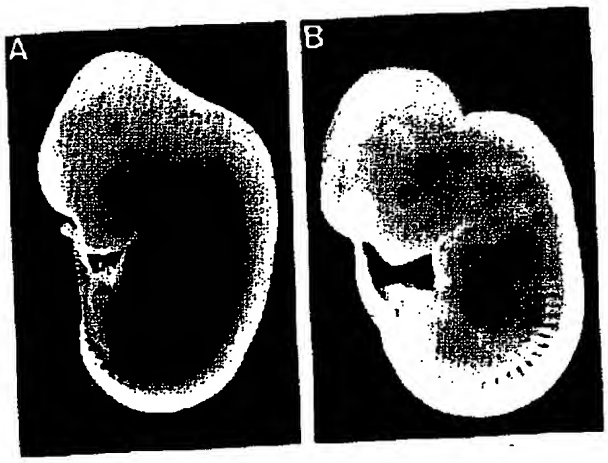


Fig. 5. Comparison of ES-generated E12.5 chimaeric embryos with targeted (A) versus randomly integrated (B) *myf-5/nlacZ* constructs.

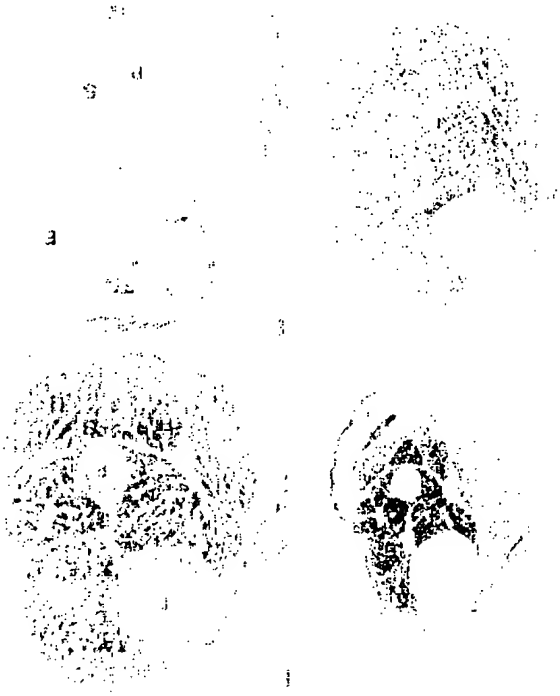
somitic and limb musculature, compared with muscle masses in the head or arches. However, it is now clear from comparison with the *nlacZ* reporter in the *myf-5* locus that there is a major quantitative difference with the transgene. This may complicate interpretation of the onset and continued expression of the transgene because of a detectability problem. We conclude that other regulatory sequences, including enhancer elements, are present elsewhere in the locus (and possibly shared with *MRF4*).

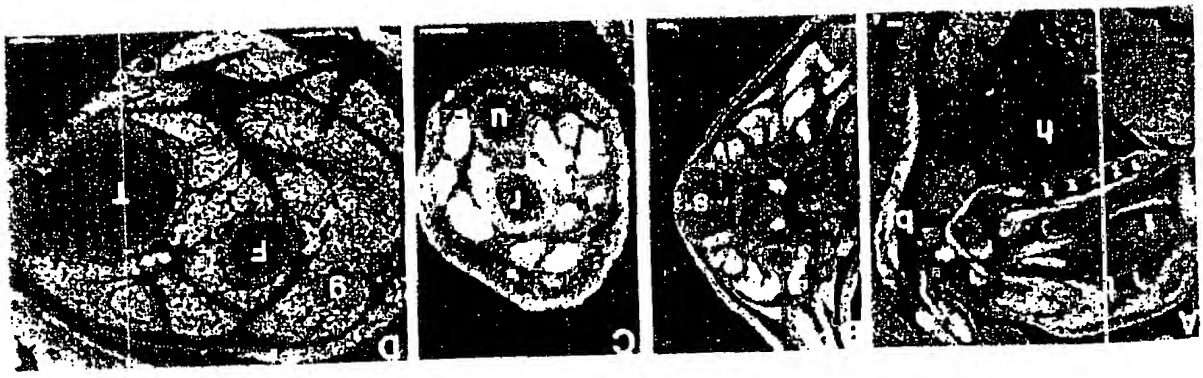
Myf-5 Expression in the Embryo

Analysis of β-galactosidase activity in chimaeric and heterozygote embryos confirms the early expression of *myf-5* in skeletal muscle as it forms. All four myogenic factor genes were shown to be transcribed in skeletal muscle and its immediate precursors, and this was thought to be the only site of expression of this gene family (Lyons and Buckingham, 1992; Pownall and Emerson, 1992) Cardiac striated muscle and smooth muscle are negative for these transcripts, and this is confirmed with the *myf-5/nlacZ* mice which we have analysed. The oesophagus presents a special case. It shows *myf-5* expression in cells which might have been smooth muscle. In fact, it was shown recently that these smooth muscle cells undergo a transdifferentiation process to skeletal muscle (Patapoutian et al., 1995). Myoid cells in the thymus express *MyoD*, *myogenin* (Grounds et al., 1992), and, as we show here, *myf-5*. These cells, which are derived from the cephalic prechordal mesoderm, have myogenic potential in culture, and myotubes are often reported in thymic tumors (Seifert and Christ, 1990; see Grounds et al., 1992). The most unexpected site of *myf-5* expression is in cells of the CNS. Detailed analyses of this phenomenon in the brain (Tajbakhsh and Buckingham, 1995) and in a subset of neuronal cells in the neural tube (Tajbakhsh et al., 1994) have been described. Transcripts of other members of the MyoD family are not detectable, and this probably accounts for the absence of myogenic conversion in vivo. Expression of the *myf-5* gene in the CNS raises the possibility that it may be acting as a bHLH regulator of a differentiation pathway other than that of skeletal muscle. Indeed, a number of observations suggest some flexibility exists between the myogenic and neurogenic differentiation programmes (Tajbakhsh et al., 1994).

Myf-5 Expression as Muscle Matures

The *myf-5/nlacZ* mice permit us to follow *myf-5* expression in muscle cell populations which will give rise to different fibre types during myogenesis. The *myf-5* gene is expressed at a high level in the embryo from E8-11.5. In the initial in situ hybridization studies transcripts were no longer detectable at E14 (Ott et al.,





that, as the β-galactosidase labelling indicates, they are present throughout the muscle masses. At later stages it is difficult to detect the endogenous transcript, but the β-galactosidase labelling remains clearly detectable several days after birth in the majority of skeletal muscles. Although this may partly reflect β-galactosidase stability, it is clear that nuclei in all labelled fibres transcribed the *myf-5* gene during foetal and, at least in some cases (Duclert et al., 1991), post-natal muscle development. It is not surprising in this context that $MyoD^{-}/\_$ mutant mice make mature skeletal muscle (Rudnicki et al., 1992). In these mice *myf-5* transcript levels remain high, and we would suggest that this is because nuclei in essentially all skeletal muscle fibres are transcribing this gene; in the absence of MyoD, transcription of *myf-5* is not down-regulated, and this factor accumulates. Indeed it would be very unlikely that mature muscle fibres develop normally in the $MyoD^{-}/\_$ mutant mice, from a small subpopulation of $myf\text{-}5^{+}$ embryonic myoblasts. In $myf\text{-}5^{-}/\_$ mutant mice, muscle appears to develop normally from the time when the *MyoD* gene is activated (Braun et al., 1992). This indicates that *myf-5* expression in $MyoD^{+}$ muscle cells is not essential, as might be expected from the low levels normally present. The question then arises of whether later low level expression of *myf-5* has any significance. Does it merely reflect transcriptional leakage from the locus at later times when the *MRF4* gene located 5′ to *myf-5* is activated? This seems unlikely since *myf-5* transcription, measured by β-galactosidase activity, decreases after birth when *MRF4* continues to be expressed at high levels. The functional significance of later *myf-5* transcription remains obscure. Although normally not essential, it may indeed provide a safety mechanism, as illustrated for the *MyoD* mutation.

It is clear from the results reported here that *myf-5* expression is not restricted to early myogenic lineages only. During the course of skeletal myogenesis, both in vitro and in vivo levels of myogenic factors fluctuate. These quantitative differences are useful when it comes to distinguishing more or less mature muscle cell/fibre populations, but where MyoD and *myf-5* are concerned, low or undetectable levels of a protein or transcript cannot be used as a criterion for distinguishing differently specified muscle cell lineages. In conclusion *myf-5* is expressed in all skeletal muscle types, irrespective of their origin from presomitic paraxial mesoderm or cephalic prechordal or paraxial mesoderm. If *myf-5* negative precursor muscle cell populations exist in vivo, they do not appear to make a significant contribution to mature skeletal muscle in normal mice.

EXPERIMENTAL PROCEDURES

Construct

A *lacZ* plasmid (pSKT*nlacZ*) was designed (Bonnerot et al., 1987) that contained the nuclear localization signal (n) of SV40 large T antigen fused to the β-galactosidase coding sequence lacking eight N-terminal amino acids, followed by an SV40 polyA (adenylation) sequence. This *nlacZ* construct was introduced into a fragment of the mouse *myf-5* gene ($(129 \times t^{AE5})$ F1; Braun et al., 1992) such that 5.5 kb of 5′ flanking sequence extending from an upstream ScaI site to a ScaI site 13 amino acids after the *myf-5* ATG, preceded the *nlacZ* sequence. The neomycin gene under control of the thymidine kinase promoter (*tkNeo*), obtained from pMC1*Neo*pA (Stratagene), was introduced into the construct immediately after the *nlacZ*, as was the herpes virus thymidine kinase gene with its promoter (*HSV-TK*) obtained from pMC1TK (a gift from M. Capecchi; Mansour et al., 1988), for positive and negative selection of clones, respectively. Between the two selectable marker genes a BalI fragment, 1,050 bp in length, covering part of the first exon and intron of the *myf-5* gene, served as the 3′ homology fragment. Introduction of the *nlacZ/tkneo* sequences into the *myf-5* locus by homologous recombination using the plasmid M5HR resulted in a deletion of 154 bp from amino acids 13 to 65 (Fig. 1). The plasmid was linearized with Sac II for electroporation.

Electroporation Into ES Cells and Screening of Clones

D3 ES cells (a gift from M. LeMeur, R. Kemler (Gossler et al., 1986)) were grown on feeder layers of primary embryonic fibroblasts prepared from E12.5–14.5 mouse embryos containing a neomycin transgene (a gift from R. Kemler; SV129 *Neo*), in the presence of DMEM medium, 10% foetal calf serum, and 10% newborn calf serum (Robertson, 1987). ES cells were passaged 24–48 hr prior to electroporation, trypsinised (0.25%), resuspended at a density of 2×10^{7} cells in phosphate-buffered saline (PBS, GIBCO) without Ca^{2+}/Mg^{2+}, and mixed with 20 μg linearised DNA. After 5 min at room temperature, the cells were electroporated at 960 μF, 200 mV (time constant between 11–15 msec) and incubated at room temperature for a further 10 min. Cells were then spread onto fresh feeder layers in 10-cm Falcon dishes. After 24 hr, G418 was added (300 μg/ml dry weight) followed by gancyclovir (GANC) at 2 μM concentration at 48 hr. In some experiments, GANC concentration was dropped to 1 μM after 4 days of selection.

After 10–12 days of selection, individual colonies were picked with a P200 pipetman and disrupted by pipeting in a 96-well dish containing 30–50 μl medium. Polymerase chain reaction (PCR) analysis was carried out on DNA prepared from pools of 12 clones essentially as described (Kim and Smithies, 1988). Half of the colony was used for the PCR pool, while the other half was grown on feeder cells in 96-well dishes. The 5′ Neo specific primer used was NP3:GGTATCGCCGCTCCCGATTCGC. The 3′ PCR primer (M5P5:GGACAGTAGATGCTGTCAAAGCTGC) was derived from a BalI–BglII fragment present in the *myf-5* gene, 3′ to and outside the region of homology. A control construct

for PCR analysis was made with an additional 150 bp at the 3′ end, containing the 3′ PCR primer. Amplified DNA was analysed by Southern blotting using as a probe the KpnI–BalI 3′ *myf-5* fragment. Individual clones from positive pools were rescreened by PCR. DNA from single positive clones was digested with BglI and checked by Southern analysis, using the same KpnI–BalI probe. The wild-type allele would yield a 2.8-kbp fragment while the mutated allele would reveal a 7.1-kbp fragment. The integrity of the targeting event was confirmed with probes located outside the homology region and with the *Neo* gene.

Embryo Injections

Embryos at the 8-cell stage were flushed from the oviducts of superovulated (C57BL6 × SJL-J) F1 mice and stored in Whitten medium (Whitten, 1971) until just prior to injection. The morula were decompacted for 20–40 min in PBS without Mg^{2+}/Ca^{2+} at 37°C then placed in a microdrop of PBI (Whittingham and Wales, 1969) while the ES cells were maintained in a separate microdrop of DMEM/10% FCS/100 mM Hepes and 100 mM β-mercaptoethanol, all under oil. Morula began to compact more rapidly in DMEM (10 min) than PBI (1.5–2 hr). Eight to ten ES cells were placed between the cells of the morula, and embryos were replaced in Whitten microdrops at 37°C for 1–4 hr until they were implanted into the oviducts of pseudopregnant (C57BL6 × CBA) F1 fosters which were plugged the same day. Alternatively, injected morula were allowed to form blastocysts after overnight incubation then implanted into the uterus of E2.5 pseudopregnant foster mothers. The number of developing embryos and percent chimaerism was highly dependent on the quality of the ES cells and the number injected. In some cases with later passage cells, injection of 8–10 cells resulted in higher numbers of abnormally developed embryos; therefore fewer cells (5–6) had to be injected.

Embryo Dissections, Staining, and In Situ Hybridizations

Embryo staging was based on transfer of injected embryos to the oviduct E0.5 or uterus E2.5 of foster mothers (Rugh, 1990). More precise staging was based on somite counts. For β-galactosidase staining (Sanes et al., 1986), embryos were rinsed once in PBS and fixed in 4% paraformaldehyde for 30 min to overnight depending on the size of the embryo. After rinsing 3 times in PBS (total 15 min), embryos were stained in a solution containing 4 mM each of potassium ferrocyanide, potassium ferricyanide, 2 mM $MgCl_2$, 400 μg/ml X-gal, and 0.02% NP40 in PBS at 32°C overnight with gentle agitation. X-gal stocks were prepared in dimethylsulfoxide at 40 mg/ml and stored at −20°C. Photographs of whole-mount stained embryos were taken with an Olympus SZH10 stereomicroscope.

Stained embryos were embedded in paraffin or resin for sectioning. Five- and 2-μm sections were obtained using a microtome. Sections were stained with Safranin O (0.0025%) in some cases to improve morphological resolution. In situ hybridizations were carried out on paraffin sections with a *myf-5* specific probe (Ott et al., 1991) using two radionucleotides (Tajbakhsh and Houzelstein, 1995) and 10 days exposure time. Sections were examined and photographed using a Zeiss Axiophot microscope.

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